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ANTI-INFLAMMATORY PROPERTIES OF *CANTHARELLUS CIBARIUS* FROM *IN VITRO* CULTURE ENRICHED IN ZINC

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Abstract: *Cantharellus cibarius* Fr. (*Basidiomycota*), commonly known as Chanterelle, is one of the most valued and currently most often collected species of edible mushrooms in Europe, Asia, Africa, and the northern USA. *Cantharellus* genus (chanterelles) includes seventy species, seven of which are noted in Europe. Due to the valuable and biologically active metabolite content in the fruiting bodies, *C. cibarius* was selected for the study. The combinations of carbohydrates, amino acids, unsaturated fatty acids, and vitamins revealed the nutritional, in particular, anti-inflammatory properties of this species. Zinc (Zn) is one of the microelements present in significant quantities in the fruiting bodies and biomass from *in vitro* cultures of edible mushrooms. Zn plays a crucial role as an anti-inflammatory agent. This paper presents a study on investigation of the pro- or anti-inflammatory properties of mushroom extracts from fruiting bodies and biomass from *in vitro* culture of *C. cibarius* enriched in Zn. As an additive to the modified Oddoux liquid medium, on which *in vitro C. cibarius* cultures were grown, the following two compounds were used: zinc sulfate and zinc hydroxyaspartate. In order to compare which complex will most effectively increase the anti-inflammatory activity of *C. cibarius*, inorganic and organic compounds were used. Both zinc salts were used in such quantities that the calculated additive amount of zinc into the culture media was the same. Control cultures were represented by *in vitro* cultures on Oddoux medium grown without the supplementation of zinc compounds. In addition, we studied which source of zinc is best for enhancing anti-inflammatory properties in A549 (Human Lung Epithelial Carcinoma, ATCC) cell models activated with lipopolysaccharide (LPS) to induce inflammation. Results of the study showed the important role of zinc during inflammation in the lung epithelial cells. *Cantharellus cibarius in vitro* cultures with high ability to accumulate zinc enabled the precise application of zinc compounds at a known concentration that may influence their immuno-modulatory properties.

Keywords: *Cantharellus cibarius*, mushroom *in vitro* cultures, zinc, anti-inflammatory properties

Abbreviations: AHR – aryl hydrocarbon receptor, COX-2 – cyclooxygenase-2, LPS – lipopolysaccharide, cPGES – prostaglandin E synthase

From ancient times, people have used mushrooms for both medicinal and dietary purposes. *Cantharellus cibarius* Fr. (*Basidiomycota*), commonly known as chanterelle, is one of the most valued and currently most often collected species of edible mushrooms in Europe, Asia, Africa, and the northern USA. *Cantharellus* genus (chanterelles) includes seventy species, seven of which are noted in Europe. Due to the valuable and biologically active metabolite content in the fruiting bodies, *C. cibarius* was selected for the study. Some of these active compounds, which can be isolated, especially from *C. cibarius in vitro* culture, can be used as dietary supplement or medicines (2-5).

The fruiting bodies of *C. cibarius* are composed of both saturated and mono- and polyunsaturated fatty acids, whose content is 22.63%, 23.29%, and 54.08% (% of total lipids), respectively (6). Oxidation of the free linoleic acid in the process by catalyzed lipoxygenase and hydroperoxide lyase in

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the fruiting bodies of *C. cibarius* results in the formation of 1-octen-3-ol responsible for the specific odor of this specie (6). A group of compounds present in the fruiting bodies and the biomass from *in vitro* cultures of *C. cibarius* – demonstrating an anti-inflammatory activity and includes indole compounds (L-tryptophan, 5-hydroxytryptophan, serotonin, melatonin, 5-methyltryptophan, and tryptamine) (7, 8). The fruiting bodies of this species contain a high content of phenolic acids, in particular hydroxybenzoic acid, which exhibits potent anti-inflammatory properties (3, 9-11).

Numerous studies of chemical composition demonstrated that the fruiting bodies of *C. cibarius* are also a source of vitamins A, D, E, and C. Moreover, this specie is also a rich source of ergocalciferol (vitamin D_2), whose content is high even after several years' storage of dried fruiting bodies – 1.43 µg/g on average (12–14). The bioavailability of ergocalciferol from the fruiting bodies is 55-99% and increases in proportion to the fat content in the diet (15). The presence of tocopherol (vitamin E) was demonstrated in the fruiting bodies of *C. cibarius* (16).

Mushrooms are characterized by high ability to accumulate elements. One of microelements present in significant quantities in the fruiting bodies and biomass from *in vitro* cultures, which play a key role as an anti-inflammatory agent, is zinc (17).

The aim of the study was to investigate the pro- or anti-inflammatory properties of mushroom extracts from fruiting bodies and biomass from *in vitro* culture of *C. cibarius* enriched in zinc. In order to compare which complex will most effectively increase the anti-inflammatory activity of *C. cibarius*, we used inorganic and organic compounds. As an additive to the modified Oddoux liquid medium, on which *in vitro C. cibarius* cultures were grown, were used zinc(II) ions in the following two compounds: zinc sulfate and zinc hydroxyaspartate. Both zinc salts were used in such quantities that the calculated additive amount of zinc ions into the culture media was the same. Control cultures were represented by *in vitro* cultures on Oddoux medium grown without the addition of zinc compounds. The next goal of this study was to find which source of zinc is best for enhancing anti-inflammatory properties in A549 (Human Lung Epithelial Carcinoma, ATCC) cell models activated with lipopolysaccharide (LPS) to induce inflammation.

MATERIALS AND METHODS

Reagents and standards

Zinc hydroaspartate was obtained from Farmapol (Poland). Zinc sulfate came from OUM-7, Łódź. In the experiment, concentrated mineral acids; HCl, HNO_3, and 30% H_2O_2 solution were used, all were Suprapur® grade (Merck, Darmstad, Germany). Of about 1 and 10 µg/mL standard solutions of zinc were prepared by appropriate dilution of 1000 µg/mL stock standard solution (Okręgowy Urząd Miar, Łódź, Poland).

Ethanol was from Sigma-Aldrich St. Louis (USA); HClO solution was manufactured by Unilever (Hungary); n-hexane, chloroform, and methanol were purchased from Merck (Germany). Quadruple distilled water with conductivity lower than 1 µS/cm was prepared in S2-97A2 quartz distilling unit (Chemland, Stargard Szczeciński, Poland).

Table 1. Analysis of Zn concentration in cells and medium in A549 cells, with and without an inflammatory agent LPS from *E. coli* (µg/mL) after the incubation with the extracts from biomass and fruiting bodies of *Cantharellus cibarius*.

Mushroom material	Cells	Cells + LPS	Medium	Medium + LPS
	[µg/mL]			
Control	0.92 ± 0.09[a]	1.45 ± 0.04[a,b]	0.45 ± 0.05[a,b]	0.37 ± 0.03[a,b]
Extracts of *C. cibarius* biomass	1.25 ± 0.08[a]	2.96 ± 0.04[a,b]	0.53 ± 0.03[a,b,c]	0.21 ± 0.00[a,b,c]
Extracts of *C. cibarius* biomass with zinc hydroaspartate	2.45 ± 0.08[a]	3.22 ± 0.08[a,b]	0.42 ± 0.02[a,b]	0.31 ± 0.01[a,b]
Extracts of *C. cibarius* biomass with zinc sulfate	2.01 ± 0.08[a]	3.43 ± 0.10[a,b]	0.63 ± 0.03[a,b,c]	0.32 ± 0.03[a,b,c]
Extracts of fruiting bodies of *C. cibarius*	1.16 ± 0.11[a]	2.12 ± 0.05[a,b]	0.51 ± 0.04[a,b,c]	0.31 ± 0.01[a,b,c]

Data are presented as the mean ± SD (standard deviation); n = 4 repetitions. Tukey-Kramer test was used to reveal the differences between paired groups of elements in rows, the same letters (a, b, c) are marked for which the content differences are statistically significant (for p values < 0.05), (GraphPad InStat).

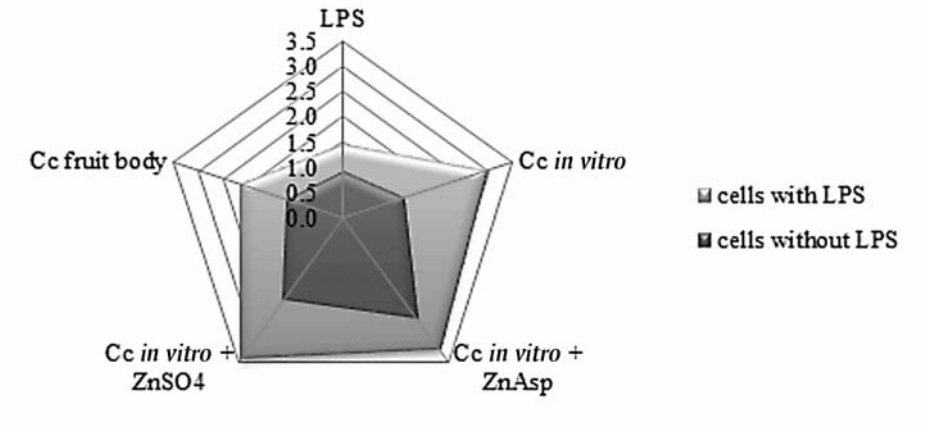

Figure 1. Radar chart showing the distribution of Zn concentration in A549 line cells and in the cells induced with an inflammatory agent LPS from *E. coli* (C – control; LPS – LPS-activated samples; Cc *in vitro* – extract of *C. cibarius* biomass from *in vitro* culture; *Cc in vitro* + ZnAsp – extract of *C. cibarius* biomass from *in vitro* culture enriched with zinc hydroaspartate; Cc *in vitro* + $ZnSO_4$ – extract of *C. cibarius* biomass from *in vitro* culture enriched with zinc sulfate; Cc wild – extract of fruiting bodies of *C. cibarius*)

Mushroom materials

The explants for the generation of the mushroom *in vitro* cultures were taken from fresh fruiting bodies of *C. cibarius*. Initial mushroom materials were harvested from natural state (mixed forests in southern Poland) during 2014–2015. Prof. Bożena Muszyńska performed the taxonomic identification of collected young mushroom fruiting bodies. Representative voucher specimens were deposited at the Department of Pharmaceutical Botany, Jagiellonian University Medical College, Kraków, Poland. In order to conduct the experiment, *in vitro* cultures of *C. cibarius* were grown on a modified liquid medium with composition according to Oddoux and on the same medium supplemented with zinc(II) ions in inorganic (zinc sulfate) and organic (zinc hydroxyaspartate) compounds (18).

In vitro* cultures of *C. cibarius

The fresh explants (the fragments of fruiting bodies of *C. cibarius*) were degreased with 70% ethanol for 15 seconds, sterilized for 2 minutes with 15% with sodium hypochlorite and rinsed repeatedly in sterile distilled water. Then, clear *C. cibarius* fragments were transferred onto the solid Oddoux medium (laminar airflow) (18). In the next step, to achieve maximum efficiency in fungal biomass growth, the mushroom cultures were transferred to the modified liquid Oddoux medium (starting inoculum transplanted from the solid medium culture was 0.1 g). To prepare the experimental cultures, the obtained biomass was passaged into Erlenmeyer's flasks (500 mL) containing the liquid medium (250 mL) and conducted at 25 ± 2°C under a 16-h photoperiod (900 lx/8 h dark). The biomass was obtained from the cultures grown on the Oddoux medium (control), and on the same medium but with the addition of zinc hydroaspartate at a concentration of 100 mg/L, and zinc sulfate(VI) at a concentration of 87.23 mg/L. The applied concentrations of both compounds contained the same content of zinc(II) ions (20 mg/L). Five cultures of *C. cibarius* were derived for both variants. After four weeks, since the initiation of *in vitro* cultures on liquid medium, the biomass was separated from the medium and rinsed three times with fourfold distilled water. The resulting biomass was frozen and then dried *via* lyophilization (lyophilizer Freezone 4.5, Labconco; temperature: -40°C).

For the preparation of *C. cibarius* methanolic extracts, the lyophilized mushroom materials such as the fruiting bodies and biomass from *in vitro* culture were portioned and weighed (5 g of each sample), then ground in a mortar and subjected to extraction with petroleum ether in percolators in order to remove the lipid fraction according to the procedure developed by Muszyńska et al. (19, 20). The remaining degreased biomass was dried and again subjected to extraction with methanol in a percolator for 24 h (kept in the dark). The obtained extracts were concentrated by distillation in a vacuum evaporator (Büchi, Germany) under reduced pressure (200 mbar) at 40°C. The resultant extracts were dissolved in distilled water (1 g of dry extract to 1 mL of water), then filtered through bacteriological 0.2-μm syringe filters, and then diluted to

desired concentrations. The prepared extracts were stored at 4°C until use.

Cell cultures

Human lung carcinoma epithelial A549 cells (ATCC CCL-185) were cultured using F 12K Medium with 10% Fetal Bovine Serum with the addition of antibiotics: penicillin (100 IU/mL) and streptomycin (100 mg/mL), (ATCC, Manassas, VA, USA). Cells were seeded into 6-well plates (Sarsted, Nümbrecht, Germany) at a density of 5×10^5 cells/well in 1 mL of medium and carried at 37°C in a humidified atmosphere with 5% CO_2. During the experiment, the morphology of cells was investigated by an inverted light microscope (Olympus, Tokyo, Japan). In addition, the viability of cells was controlled with Trypan Blue Exclusion Test. No cytotoxic effects or apoptosis was observed in A549 cells.

After 24 h after seeding, A549 cells were activated with 10 ng/mL lipopolysaccharide (LPS from *E. coli* 026:B6 Sigma-Aldrich, Saint Louis, MI, USA) and incubated with 20 µL/mL of *C. cibarius* extracts for 24 h. After incubation, both the media and the cells fraction were collected and then prepared for the determination of zinc content and protein quantification by using Western blot technique.

Western blot – protein quantification

Cell lysates were prepared with M-PER Buffer (Thermo Scientific, USA) with addition of proteases (Calbiochem, Merck, Germany) and phosphatase inhibitors (Cayman Chemical, AnnArbor, MI, USA). Total protein concentration was determined using the Bradford reaction. Lysate aliquots (50 µg) were solubilized in a Laemmli buffer with 2% 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA) and subjected to 10% SDS-polyacrylamide gel electrophoresis as described earlier (21, 22). The following antibodies were used: COX-2 (1 : 1000), cPGES diluted (1 : 500), AHR (1 : 500) and β-actin (1 : 1000), and secondary Easy Blot anti-rabbit IgG (HRP) (1 : 1000) diluted in Signal + for Western blot (GeneTex, Irvine, CA, USA). Proteins were detected using a Clarity Western ECL Luminol Substrate western blotting detection kit (Bio-Rad). Using a ChemiDoc Camera with Image Lab software (Bio-Rad), the integrated optical density of the bands was quantified.

Determination of zinc with the FAAS method

The material obtained in the experiment (medium after the culture of A549 cells, homogenized fractions of A549 cells, and extracts from biomass and fruiting bodies of *C. cibarius*) was subjected to microwave digestion in a closed system (Ertec, Magnum II). For this purpose, 1 mL of the sample was collected for the media, 100 µL of the sample for cell fraction, and 500 µL of the sample for the extracts of *C. cibarius*; then, they were transferred to a Teflon vessel. A total of 2 mL of concentrated nitric acid(V) and 1 mL of H_2O_2 were added, and the whole mixture was placed in the mineralizer (MS Spectrum). Microwave digestion comprised the following three main stages:

I – heating, duration time 10 minutes, power 300 W;

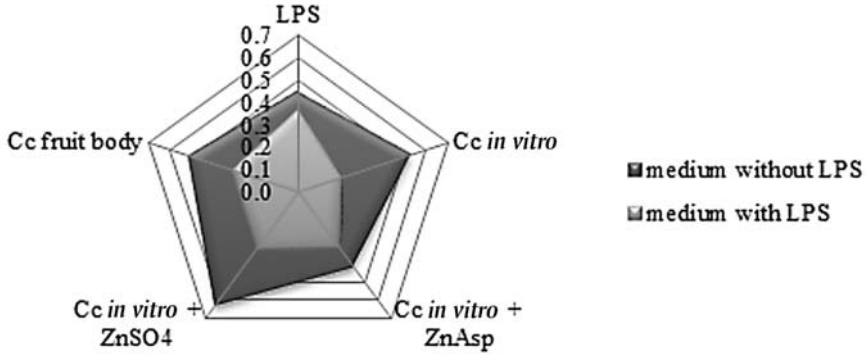

Figure 2. Radar chart showing the distribution of Zn concentration in post-culturing medium from A549 line cell, and those induced with an inflammatory agent LPS from *E. coli* (C – control; LPS – LPS-activated samples; Cc *in vitro* – extract of *C. cibarius* biomass from *in vitro* culture; Cc *in vitro* + ZnAsp – extract of *C. cibarius* biomass from *in vitro* culture enriched with zinc hydroaspartate; Cc *in vitro* + $ZnSO_4$ – extract of *C. cibarius* biomass from *in vitro* culture enriched with zinc sulfate; Cc wild – extract of fruiting bodies of *C. cibarius*)

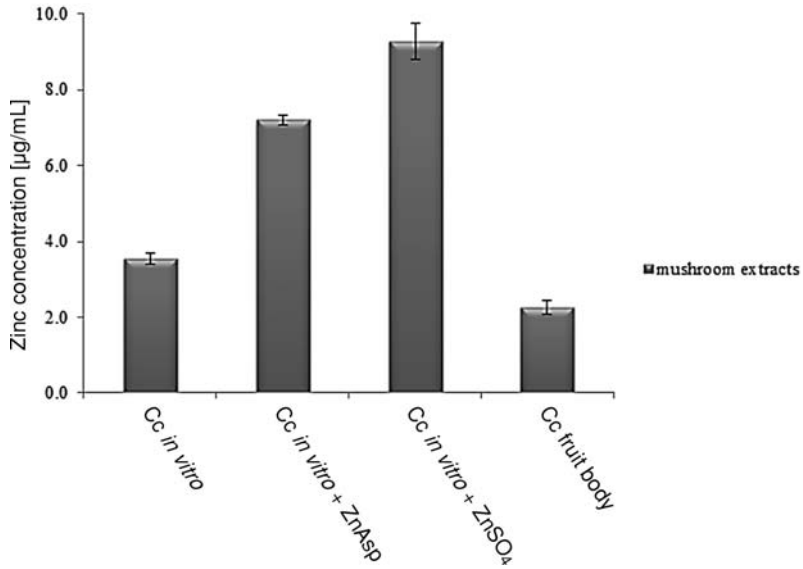

Figure 3. Zinc concentration (µg/mL) in *Cantharellus cibarius* extracts. Cc *in vitro* – extract of *C. cibarius* biomass from *in vitro* culture; Cc *in vitro* + ZnAsp – extract of *C. cibarius* biomass from *in vitro* culture enriched with zinc hydroaspartate; Cc *in vitro* + $ZnSO_4$ – extract of *C. cibarius* biomass from *in vitro* culture enriched with zinc sulfate; Cc fruit body – extract of fruiting bodies of *C. cibarius*

II – heating, duration time 20 minutes, power 600 W;

III – cooling, duration time 20 minutes.

The digested sample was transferred to a quartz crucible and heated at a temperature of 60°C for 45 minutes in order to evaporate excess of reagents. Then, the obtained sample was quantitatively transferred to a volumetric flask with a capacity of 5 mL. Redistilled water was used to transfer the evaporated samples to the flasks and diluted to 5 mL.

The samples were mineralized in triplicate. The quantitative analysis of Zn content in the resulting material was performed using atomic absorption spectrometry (FAAS) (Spectrometr AA ICE 3000).

Statistical analysis

Values are presented as the means ± SD. All experiments were performed four times. Using one-way ANOVA with Tukey-Kramer *post hoc* method of multiple comparisons, statistical analysis of the data was performed. The value $p < 0.05$ was accepted as the level of statistical significance. Chemometric tools were used in order to facilitate the analysis and interpretation of the data obtained in the experiment; these included the two main methods: cluster analysis (CA) and principal component analysis (PCA). Cluster analysis enabled the identification of groups of similar objects that were described by four parameters (Zn, COX-2, cPGES2, AHR). PCA as a method of calculation allowed the reduction of the size of the data and demonstration of the correlations between the objects in two-dimensional spaces. Calculations were performed using (GraphPad InStat, USA), and Statgraphics Centurion XVII. Statistical significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

AAS method was selected for trace analysis of zinc in mushroom material, because its instrument characterizes with high sensitivity, accuracy, and precision of the analysis.

An average content of zinc in the cell lysates after the incubation with *C. cibarius* extract was 1.16 µg/g, while zinc concentration in LPS-activated cells increased to 2.12 µg/g. After the incubation of A549 cells with *C. cibarius* extracts enriched with zinc hydroaspartate, the content of this element was 2.45 µg/g. The cells activation with LPS and incubation with *C. cibarius* enriched with $ZnSO_4$ resulted in an increase in Zn content in the cells to an average value of 3.43 µg/g (Table 1). It was observed based on the analysis of Zn, in which the addition of mushroom extract in any case increases the concentration of this element in A549 cells (Table 1, Figs. 1, 2).

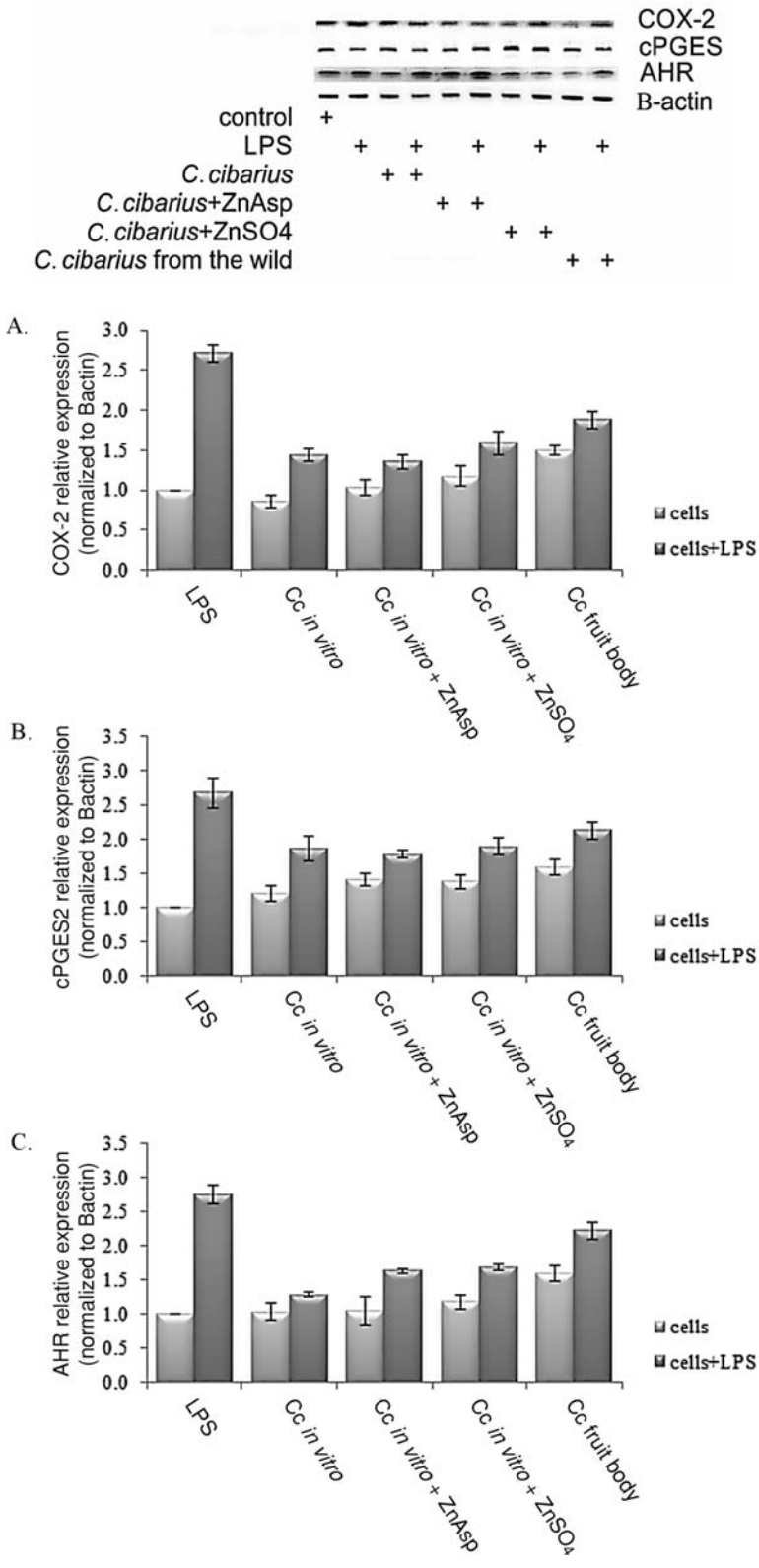

Figure 4. Relative expression of COX-2 (A), cPGES (B), and AHR (C) in A549 supplemented with *Cantharellus cibarius* extracts and activated with LPS from *E. coli*. C – control; LPS – LPS-activated samples; Cc *in vitro* – extract of *C. cibarius* biomass *from in vitro* culture; Cc *in vitro* + ZnAsp – extract of *C. cibarius* biomass *from in vitro culture* enriched with zinc hydroaspartate; Cc *in vitro* + $ZnSO_4$ – extract of *C. cibarius* biomass *from in vitro culture* enriched with zinc sulfate; Cc fruit body – extract of fruiting bodies of *C. cibarius*

Activation of lung epithelial cells with LPS resulted an increase in Zn accumulation in the cells. This fact is also confirmed by the amount of Zn remaining in the medium after the cell culture. In each of the discussed cases, it is lower for cells induced with inflammatory agent. In turn, the lowest concentration of Zn was observed both for the cells themselves, as well as for those induced with LPS for the cultures with an addition of the extract derived from fruiting bodies of *C. cibarius*. Considering Zn content in the extracts (Fig. 3) introduced to the A549 cells culture, an increasing amount of Zn in the extract added to the cell culture increases the degree of Zn accumulation in A549 cell lines; this effect is enhanced in case of the cells induced with an inflammatory agent.

Another tendency was observed analyzing the results obtained for an expression of proteins COX-2, cPGES2, and AHR. The highest expression of proinflammatory proteins and AHR was observed in the LPS-activated A549 cells (Fig. 4). The addition of the extracts from *in vitro* cultures of *C. cibarius* obtained on media enriched with zinc hydroaspartate or $ZnSO_4$, which were characterized by a high content of zinc to A549 cells, causes a reduced protein expression in A549 cells activated with LPS (Fig. 4).

Chemometric analysis

Chemometric tools were used in order to obtain further information and to present the results in a simple and accessible way. This allowed to separate a significant information about the existing relationships between the measured variables (Zn, COX-2, cPGES2, AHR) and the objects (A549 cells, A549 cells with LPS). An unequivocal interpretation of the data and determination of the systematic changes in case of culturing of the cells is complicated; therefore, chemometric methods were used for this purpose. Initially, the cluster analysis (CA) method was applied, which is based on the assumption that the objects are recognized as similar if the distances between them are small, and consequently, their position in the multidimensional space of the variables is close. As a result, the examined set of objects may be divided into groups (the so-called clusters) that are characterized by a similar variability (23-26). Squared Euclidean method was used as a measure of distance. Then, the distance between the clusters was calculated according to the Ward's method. This method is based on the concept of analysis of variance, characteristics of similarity (Zn, COX-2, cPGES2, AHR), and objects (cells). The results are presented graphically in the form of so-called trees – dendrograms (Figs. 5, 6).

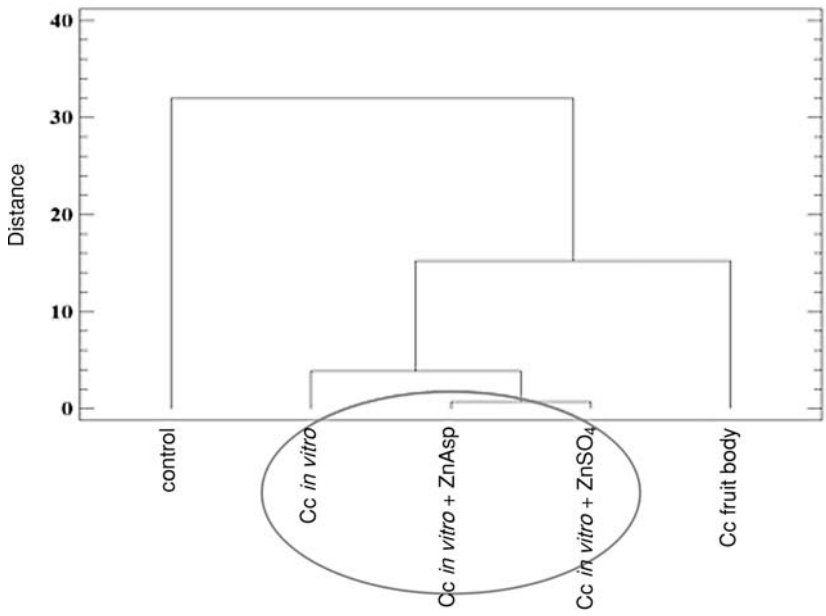

Figure 5. Dendrogram – similarity between the analyzed objects – A549 cells with the addition of mushroom extracts (Squared Euclidean metric, Ward's algorithm). C – control; LPS – LPS from *E. coli*-activated samples; Cc *in vitro* – extract of *C. cibarius* biomass *from in vitro* culture; Cc *in vitro* + ZnAsp – extract of *C. cibarius* biomass *from in vitro culture* enriched with zinc hydroaspartate; Cc *in vitro* + $ZnSO_4$ – extract of *C. cibarius* biomass *from in vitro culture* enriched with zinc sulfate; Cc fruit body – extract of fruiting bodies of *C. cibarius*

The x-axis on the dendrogram corresponds to both the analyzed objects to which mushroom extracts were added (Fig. 5) and the examined elements (Fig. 6), while the y-axis represents the distance between objects (23-26).

The presence of subgroups characterized by similar variability was observed based on the analysis of similarity (CA – Fig. 5). This group contains the objects to which the extracts from *C. cibarius in vitro* cultures were added. Furthermore, the highest similarity exists between the cells with the addition of extracts from *in vitro* cultures carried out in media enriched with zinc hydroaspartate and zinc sulfate, as evidenced by the short arms of the dendrogram. The highest degree of Zn accumulation was demonstrated just for these cells. Also an apparent lack of similarity for cells containing the extracts from fruiting bodies of *C. cibarius* and the cells with the addition of the extracts from *in vitro* cultures of this mushroom species may be noticed, which indicates the localization on a separate arm of the dendrogram. Considering the second dendrogram (Fig. 6) showing the similarity between the analyzed variables, 3 clusters were separated. The first cluster includes the A459 cells with or without an inflammation agent, in which Zn concentration was analyzed, while the second cell group comprises the lung epithelial cells, in which the proteins COX-2, cPGES2, and AHR expression was analyzed. In turn, the last third group includes the LPS activated cells. All of these clusters showed a high correlation in each group, but concurrently, they highly differed from one another as may be concluded from the long arms of the dendrogram. The observed relations confirm the earlier results of the occurrence of similar tendencies concerning the concentrations of Zn and proteins in groups of cells with an inflammatory agent and without this agent.

As a supplement to the method of cluster analysis, the method of principal components analysis (PCA) was also used in this study. This method allows to reduce the number of the examined variables required to describe the existing interactions (23-26). On this basis, 98% of changes in the analyzed data set can be described using the first two principal components (PC1 and PC2), thereby, further analysis did not consider the other elements. The resulting variables PC1 and PC2 are a linear combination of input variables multiplied by the loads assigned to them. The load corresponds to the level of particular variable saturation. In practice, this means that this factor is correlated with the

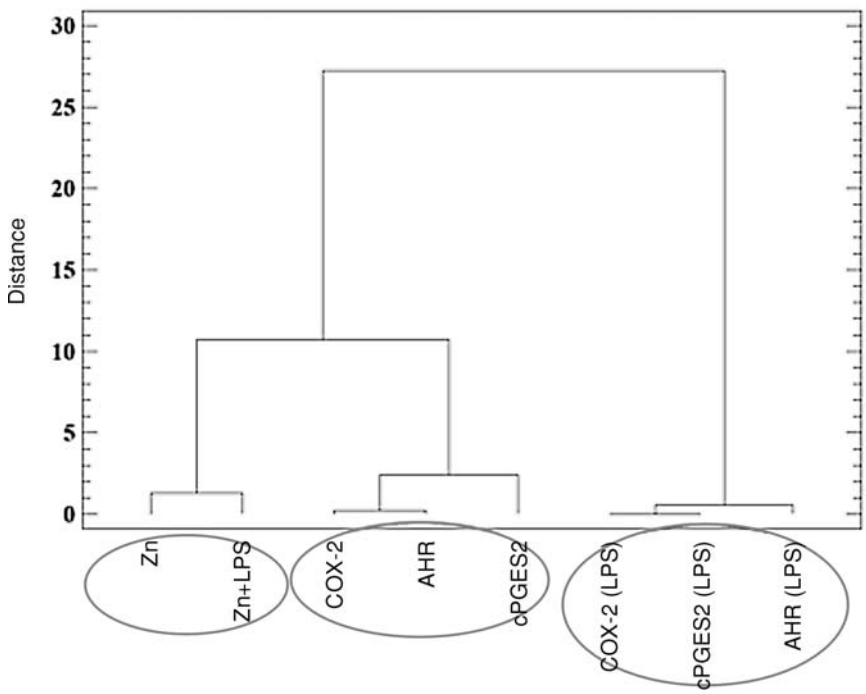

Figure 6. Dendrogram – similarity between the analyzed variables – the content of Zn, COX-2, AHR and cPGES2 in A549 cells with and without LPS from *E. coli* factor (Squared Euclidean metric, Ward's algorithm). COX-2 – cyclooxygenase 2, AHR – aryl hydrocarbon receptor, cPGES2 – prostaglandin E2 synthase

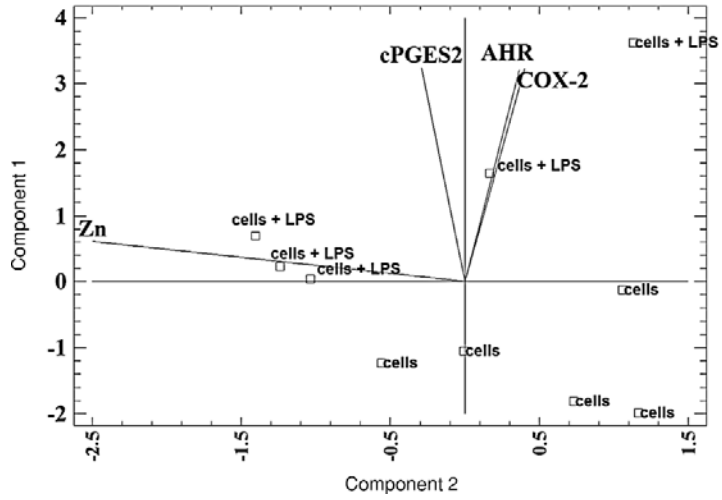

Figure 7. Biplot graph creates a two-dimensional space, shows correlation between the concentrations of zinc, COX-2, cPGES2, and AHR in cells with and without LPS

Table 2. Factor loads for two first main principal components (PC1 and PC2).

Element	Principal component 1	Principal component 2
Zn	0.108	-0.971
COX-2	0.573	0.155
cPGES2	0.576	-0.113
AHR	0.571	0.141

coefficient corresponding to the output variables. The highest effect on the change of the main component is noted for the component whose load is the highest. Sizes of the loads obtained for the two main components are presented in Table 2. On this basis, the variables such as COX-2, cPGES2, and AHR had an effect on the size of PC1 to the highest degree. Accordingly, PC2 component can be related to the variables in the same way.

This procedure allows the analysis of the results in two-dimensional space, which was created based on the principal components. Based on the obtained Biplot graph (Fig. 7), the area, which is characterized by the increased content of Zn and expression of proteins (guide lines facing up), contains the objects – LPS activated cells. In turn, the area with reduced content of the examined variables contains the cells without LPS, which confirms previously observed relationship.

Biological activity of *Cantharellus cibarius* biomass from *in vitro* culture

In presented work, biologically activity of *C. cibarius* biomass *from in vitro* cultures showed in LPS-activated lung epithelial cells an increase (up to threefold) zinc absorption in A549 cells when compared to control. Another finding was that zinc sulfate was better absorbed than zinc hydroaspartate. The presence of mushroom extracts in the medium (obtained from *in vitro* mushroom and *in vitro* mushroom supplemented in zinc sulfate) also promotes zinc absorption. Zinc content in culture media and cell fractions obtained in our experiments ranged from 0.21 to 0.63 mg/L for culture media and from 1.25 to over 3.43 mg/L for cell fractions.

Zinc ions play important immunoregulatory role and can reduce oxidative stress in cells. However, the effectiveness of the properties of zinc strictly depend on maintaining the homeostasis, which depends on the level of environmental zinc and its bioavailability and is tightly regulated by the transport of zinc in and outside the cells and between cells' compartments. The source of easily accessible zinc may have an impact on the cells' ability for the survival and effective response against stressor agents. In active or chronic inflammatory states, the need for zinc increases. Reducing inflammation by zinc ions occurs *via* upregulation

of zinc-dependent transcription factors (A20, MTF-1) and inhibition of NF-κB activation, which results in decreased generation of pro-inflammatory cytokines. In turn, chronic zinc deficiency promotes the production of pro-inflammatory cytokines and contributes to the prolonged inflammation (27-30).

The stimulation of immune cells with LPS and other inflammation factors has an influence on the changes in cellular zinc, which is resulted by alterations in zinc transporter expression. Alterations of zinc transporter expression could potentially affect zinc homeostasis in immune cells and contribute to immune dysfunction and chronic inflammation (27, 28).

Pathways of Zn(II) transport and their dysregulation have been linked to specific diseases. The labile nature of Zn(II) bound to specific zinc finger motifs and in cluster domains, as found in metallothionein, has shown how nitric oxide and reactive oxygen species mobilize bound Zn(II) with subsequent reentry into cellular Zn(II) pools (29).

The results of our study suggest that consumption of zinc by LPS-activated A549 cells may be related to higher activity (expression) of metallothionein (MT). MT, a protein that regulates the intracellular Zn level by strongly binding free Zn, is also increased during Zn supplementation. The basal and metal-induced transcription of MT and of ZnT1 is regulated by the metal response element-binding transcription factor-1 (MTF1), which directly senses increases in intracellular Zn concentration (29, 30).

The role of MTs and its ability to protect *in vivo* and *in vitro* against cellular stressors such as free radicals is well studied, but the mechanism of cytoprotection remains unclear (29). MT is induced by inflammatory stress, and its role in inflammation is implied. Also, MT expression in the lung can be enhanced by inflammatory stimuli, suggesting that its expression correlates with inflammatory pulmonary diseases (31).

For various tissues and cell types, different content of zinc is in an optimum range. The highest concentration and also tolerance to zinc ions have specialized neurons and pancreatic β-cells. A549 carcinoma cell line is capable to survive in high zinc concentration (200 μmol); however, cells viability decreases severely to only 5% in zinc concentration 300 μM (32).

Anti-inflammatory properties of *C. cibarius* and biomass from *in vitro* cultures result from the presence of compounds such as carotenoids, flavonoids, fatty acids, indole and phenolic compounds and microelements, in particular zinc (33-35). According to our knowledge, the biological activity of *C. cibarius* extracts has not been examined so far in *in vitro* models. Good accumulation of Zn(II) ions in the biomass from *in vitro* cultures of C. *cibarius* from media enriched with that element was demonstrated in this study.

Cyclooxygenase 2 is a pro-inflammatory enzyme; the inhibition of COX-2 by non-steroidal anti-inflammatory drugs (NSAIDs) can provide relief from the inflammation and pain. NSAIDs cause many side effects; therefore, the search for natural COX-2 agonists appears to be of particular significance.

The AHR signaling pathway plays an important role in modulating the immune response in various respiratory diseases and has shown that the respiratory system is sensitive to alterations in AHR expression or function, which also suggest the potential therapeutic effect of AHR ligands (21, 36).

The results of our study concerning the repression of pro-inflammatory proteins COX-2 and cPGES in lung epithelial cells A549 activated with LPS suggest an anti-inflammatory activity of *C cibarius* extracts from biomass of *in vitro* cultures, as well as biomass from the cultures enriched with zinc.

CONCLUSIONS

Our results showed the important role of zinc during inflammation in cell models. *Cantharellus cibarius in vitro* cultures with high ability to accumulate zinc enabled the precise application of zinc compounds at a known concentration that may influence their immuno-modulatory properties. Subject related to the search for natural products, as a source of compounds with potential pharmacological and anti-inflammatory activity should be a direction for further research.

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